iScience, Volume *24*

Supplemental information

Dynein and muskelin control myosin VI delivery towards the neuronal nucleus

Matthias Kneussel, Noelia Sánchez-Rodríguez, Michaela Mischak, and Frank F. Heisler

Transparent Methods

Mouse lines

All experiments were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German Animal Welfare Act on protection of animals. The protocol was approved by the Ethical Committee of the Freie und Hansestadt Hamburg, Amt für Gesundheit und Verbraucherschutz (Permit number: V 1300/591-00.33). Generation of muskelin knockout mice was previously described (Heisler et al., 2011). B6;129/SvEv-Mkln1$^{Gt(OST448976)Lex}$/J mice were backcrossed to C57BL/6J. Snell's waltzer mice, in which homozygous mutant mice (sv/sv) contain two null mutation alleles to functionally deplete myosin VI, were obtained from The Jackson Laboratory (B6 x STOCK Tyrc-ch Bmp5se +/+ Myo6sv/J) and were on a C57BL/6J background. Samples of transgenic mice overexpressing dynamitin in brain (Tg(Thy1-DCTN2)M21Elfh) were obtained from Erika Holzbaur (Pennsylvania, PA) (LaMonte et al., 2002; Perlson et al., 2009). All mice were maintained in a pathogen-free, temperature- and humidity-controlled vivarium on a 12 h light/dark schedule. Mice had access to standard laboratory chow and water ad libitum. Both male and female mice were included in all of the experiments. In general, littermates of the appropriate genotypes were used.

Primary neuronal culture

Primary mouse hippocampal neurons were prepared from P0 newborn mice, as previously described (Heisler et al., 2014), plated at 90,000 to 110,000 cells/well onto poly-L-lysine- (Sigma, Steinheim, Germany) coated 24 well plates (Greiner Bio-One, Kremsmünster, Austria). Cells were cultured in PNGM Primary Neuron Basal Medium supplemented with PNGM-A Single Quots (PA, OA, NSF-1, L-Glutamine) (all Lonza Group Ltd., Basel, Switzerland) and 100U/mL penicillin and 100**m**g/mL streptomycin (all Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator with 5%

CO2. Cells cultured between 10 and 11 days in vitro (DIV 10-11) were used for transfection with a calcium phosphate coprecipitation method (Heisler et al., 2014) or with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cultured neurons were subjected to immunostaining at DIV 12-14.

Antibodies

The following antibodies were used for immunoprecipitation (IP) and western blotting (WB): mouse anti-Dynein Intermediate Chain (5 µg (IP) and 1:3,000 (WB), Chemicon, Hofheim, Germany); rabbit anti-Myosin VI (4 µg (IP) and 1:2,000 (WB), Sigma, Taufkirchen, Germany); rabbit anti-Myosin VI (1:200 (WB) Santa Cruz Biotechnology, Heidelberg, Germany); guinea pig anti-Muskelin (5 µg (IP) and 1:3,000 (WB), (Tagnaouti et al., 2007); rabbit anti-GluN2B (1:500 (WB), Abcam, Cambridge, UK); mouse anti NeuN (1:500 (WB), Millipore, Temecula, CA); rabbit anti-Actin (1:2,000 (WB), Sigma, Taufkirchen, Germany); mouse unspecific IgG (5 µg (IP), Sigma, Taufkirchen, Germany); guinea pig unspecific IgG (5 µg (IP), Santa Cruz Biotechnology, Heidelberg, Germany); peroxidase-conjugated goat anti-guinea pig, goat anti-rabbit and goat anti-mouse (all 1:15,000, Dianova, Hamburg, Germany). The following antibodies were used for immunofluorescence: guinea pig anti-Muskelin (1:50, (Tagnaouti et al., 2007)); goat anti-Myosin VI (1:50, Santa Cruz Biotechnology, Heidelberg, Germany); rabbit anti-Myosin VI (1:100, Sigma, Taufkirchen, Germany); CY3- or CY5-conjugated donkey anti-guinea pig or donkey anti-goat (all 1:500, Dianova, Hamburg, Germany); Alexa Fluor 488 conjugated donkey anti-guinea pig or donkey anti-goat (40µg/ml, Invitrogen, Carlsbad, CA).

Constructs

EGFP-Dynamitin has been previously described (Palazzo et al., 2001). To generate EGFP-MyoVIdn, the entire *myo VI* tail domain was amplified from rat cDNA by PCR, a-tailed and ligated into pGEM-Teasy. It was cloned as *SalI-EcoRI* fragment into

pEGFP-C2 (BD Biosciences, Heidelberg, Germany). All constructs were verified by dideoxy sequencing.

Cellular fractionation and isolation of nuclear proteins

Nuclear and cytoplasmic fractions were obtained from fresh mouse brain tissue by using a Nuclear Extract Kit (Active Motive, Carlsbad, CA). Briefly, whole brains of adult mice were sliced into 1 mm sections in ice-cold Hypotonic Buffer containing phosphatase and protease inhibitors supplemented with DTT and Detergent (Active Motive, Carlsbad, CA). A dounce homogenizer with large-clearance Teflon pestle (Sartorius Group, Goettingen, Germany) was used for homogenization until the cells were dissociated. Cells were collected by centrifugation at 850 x g for 10 min and subjected to cytoplasmic and nuclear fractionation following the manufacturers protocol (Active Motive, Carlsbad, CA).

Coimmunoprecipitation

For coimmunoprecipitation experiments, whole brains of adult mice were dissected in ice cold PBS and homogenized in IM-Ac buffer (20mM HEPES, 100mM KAc, 40mM KCl, 5mM EGTA, 5mM $MgCl_2$, pH 7.2) with freshly added proteinase inhibitor cocktail (Roche, Mannheim, Germany), 1mM PMSF, 5mM DTT and 2mM Mg-ATP (all Sigma, Taufkirchen, Germany). The homogenate was clarified by centrifugation at 1,000 x g for 10 min and the postnuclear supernatant (S1) used for the following steps. After coupling 5 µg of antibodies to magnetic Protein G Dynabeads for 4 to 5h (Invitrogen, Carlsbad, CA), 1% Triton-X-100 preincubated extracts from S1 were incubated with antibody-coupled beads overnight, followed by extensive washing steps (4-6) with IP-buffer (150mM NaCl, 50mM Tris, pH 7.5, 5mM $MgCl_2$) containing 1% Triton X-100. Bound proteins were eluted in SDS sample buffer, subjected to SDS-PAGE and analyzed by Western blotting.

Ciliobrevin D and cLTP experiments

DIV 12-14 dissociated mouse hippocampal neurons were treated with 20 µM Ciliobrevin D (Merck, Darmstadt, Germany) or 0.4% DMSO (control) (Sigma, Taufkirchen, Germany) for 80 min at 37°C in a humidified incubator with 5% CO2. For experiments involving cLTP inductions, neurons were pretreated with 20 µM Ciliobrevin D or with 0.4% DMSO (control) for 40 min. cLTP was induced by treatment with 50 µM Forskolin, 100 nM Rolipram and 100 µM Picrotoxin (Franchini et al., 2019; Otmakhov et al., 2004) (all Tocris, Wiesbaden-Nordenstadt, Germany) for 10 min in Ringer solution without Mg^{2+} (125 mM NaCl, 2,5 mM KCl, 3 mM $CaCl^2$, 33 mM D-Glucose, 25 mM HEPES, pH 7.3) (all Sigma) and in the presence of either 0.4% DMSO or 20 µM Ciliobrevin D. Neurons were then washed once with warm PBS and for cLTP recovery incubated for 30 min in Ringer solution containing Mg^{2+} (125 mM NaCl, 2,5 mM KCl, 2 mM $CaCl^2$, 33 mM D-Glucose, 1 mM $MgCl^2$, 25 mM HEPES, pH 7.3), in the presence of either 0.4% DMSO or 20 µM Ciliobrevin D. Neurons were further processed as described in immunocytochemistry section.

Immunocytochemistry

DIV 12-14 primary cultured mouse hippocampal neurons from wildtype, muskelin knockout mice or after transfection, were fixed in 4% PFA/4% sucrose (10 min) and washed in PBS prior to permeabilization with 0.25% Triton X-100 (5 min). Unspecific binding sites were blocked with 3% (w/v) bovine serum albumin (Applichem, Darmstadt, Germany) for 1 h and cells were incubated with primary antibodies overnight at 4°C. Cells were washed three times in PBS and incubated with secondary antibodies for 1 h and with TO-PRO-3 or DAPI (both 1:1,000, Thermo Scientific, Waltham, MA) if applicable, washed extensively and mounted in Aqua Poly Mount (Polysciences, Warrington, PA). For microscopy analysis an upright Laser-scanning Confocal Microscope Fluoview FV1000 with Olympus Fluoview Software Ver. 2.1.b (Olympus, Hamburg, Germany) or an inverted Leica TCS-SP2 laser

scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) was used. For double-labeling studies, a sequential scanning mode was applied.

Electron microscopy

Mice were anaesthetized and perfused with 4% PFA and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for preembedding immunohistochemistry. Sagittal vibratome sections of the hippocampus were cut into 150 nm and immersed in 2.3 mol/L sucrose in PB, overnight, at 4°C for cryoprotection. Thereafter they were subjected to two freeze-thaw cycles in liquid nitrogen to aid penetration of immunoreagents and first treated with 1% $NaBH_4$ and then with 0.3% H_2O_2 in PBS for 30 min. After rinsing in PBS, sections were blocked with 10% horse serum (HS) containing 0.2% bovine serum albumin (BSA) for 15 min and left overnight with primary antibody (guinea pig anti Muskelin (Tagnaouti et al., 2007), 1:100) in carrier containing PBS with 1% PS and 0.2% BSA. Sections were washed in PBS, incubated with biotinylated secondary antibody (Vector Labs, Burlingame, CA) diluted in carrier for 90 min. After rinsing, sections were incubated with ABC (Vector Labs), diluted to a 1:100 concentration in PBS for 90 min. Afterwards they were washed in PBS and further incubated in diaminobenzidine (DAB)-H_20_2 solution (Sigma, Taufkirchen, Germany) for 10 min. The 150 µm thick sections were postfixed with 1% OsO_4, dehydrated in an ascending series of ethanol and embedded in Epon (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Ultrathin sections were then examined with a Zeiss EM 902 (Zeiss, Göttingen, Germany).

General Statistical Analysis

The sample size was adjusted according to results of prior pilot datasets or studies that used similar methods or paradigms. Data were analyzed using Microsoft Excel, GraphPad Prism6.07 (GraphPad Software, La Jolla, CA) and IBM SPSS Statistics 22 (IBM, Armonk, NY). Prior to analysis, data were explored by using IBM SPSS

Statistics 22. Data were explored for normality within sample groups by using the Shapiro-Wilk test and for equality of variances between sample groups by using the Levene´s test. For non-parametric data, we used the Kruskal-Wallis ANOVA and the Mann-Whitney U test. Dunn´s pairwise multiple comparisons with p values adjusted using the Bonferroni correction were calculated when applicable. A two-sample Kolmogorov-Smirnov test was used to compare the distribution between sample groups. For parametric data, the independent samples t test was used. All statistical tests used, as well as the corresponding p and n values are specified in the accompanying figure legends for each experiment. In general, an error rate of $p < 0.05$ was adopted as a yardstick for statistical significance; *: $p < 0.05$ significant, **: $p < 0.01$ very significant, ***: $p < 0.001$ highly significant. All experiments were repeated at least three times using independent primary neuron cultures or mice from individual animal litters. Parametric data are reported as mean ± the classical 95% confidence intervals for the mean. Parametric data are visualized in column scatter plots showing individual data points, and with error bars representing the 95% confidence intervals for the mean. Non-parametric data are reported as median and visualized in box and whisker plots (Tukey style). Box borders indicate the 25th and 75th percentiles, horizontal lines inside boxes indicate the median, and whiskers represent values less than 1.5 times the interquartile range lower or higher than the 25th and 75th percentiles, respectively. We added red squares to indicate the mean.

Western Blot and Confocal Image Data Analysis

For evaluation of relative immunoblot signal intensities, images were acquired using a Chemo-Cam Imager ECL HR 16-3200 (Intas, Goettingen, Germany) or GE Healthcare Amersham Hyperfilm ECL (Thermo Scientific, Waltham, MA). Immunoblot signal intensities were analyzed using the ImageJ, version 1.38, software (National Institutes of Health, NIH). Intensities were then normalized, as compared to loading control signals or to self-precipitation in case of coimmunoprecipitation experiments.

Fluorescence imaging was carried out with an upright Laser-scanning Confocal Microscope Fluoview FV1000 with Olympus Fluoview Software Ver. 2.1.b (Olympus, Hamburg, Germany) or an inverted Leica TCS-SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) using a 63x objective. For simultaneous multiple-channel fluorescence, images were taken in a sequential channel recording mode. Confocal images from multiple individual cells used for statistical analysis were obtained using identical photomultiplier values throughout each experiment. All experiments were at least replicated three times using different culture preparations or animal litters. Images were saved as overlay TIF-files and further processed offline and analyzed using MetaMorph 7.1 (Molecular Devices, San Jose, CA). First regions of interests (ROIs) were defined using the ROI tool. Overlay TIF-files were separated in green, red or blue channels using the “color separate” function. ROIs were transferred from overlay to each channel using the “transfer region” function. For definition of image thresholds, brightness was adjusted using the “Inclusive thresholding state” function. Fluorescence intensity and area measurements were performed using the “Integrated Morphometry Analysis” function. Intensity profiles along dendrites were created with the “line scan” function and correlation scatter plots as well as calculation of Pearson´s correlation coefficient was performed with MetaMorph “Correlation Plot” function. The percentage of area of colocalized signal A over B or vice versa, were calculated using the “Colocalization“ function of MetaMorph.

Supplemental References

Franchini, L., Stanic, J., Ponzoni, L., Mellone, M., Carrano, N., Musardo, S., Zianni, E., Olivero, G., Marcello, E., Pittaluga, A.*, et al.* (2019). Linking NMDA Receptor Synaptic Retention to Synaptic Plasticity and Cognition. iScience 19, 927-939.

Heisler, F.F., Lee, H.K., Gromova, K.V., Pechmann, Y., Schurek, B., Ruschkies, L., Schroeder, M., Schweizer, M., and Kneussel, M. (2014). GRIP1 interlinks N-cadherin and AMPA receptors at vesicles to promote combined cargo transport into dendrites. Proc Natl Acad Sci U S A 111, 5030-5035.

Heisler, F.F., Lee, H.K., Gromova, K.V., Pechmann, Y., Schurek, B., Ruschkies, L., Schroeder, M., Schweizer, M., and Kneussel, M. (2014). GRIP1 interlinks N-cadherin and AMPA receptors at vesicles to promote combined cargo transport into dendrites. Proc Natl Acad Sci U S A 111, 5030-5035.

LaMonte, B.H., Wallace, K.E., Holloway, B.A., Shelly, S.S., Ascano, J., Tokito, M., Van Winkle, T., Howland, D.S., and Holzbaur, E.L. (2002). Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration. Neuron 34, 715-727.

Otmakhov, N., Khibnik, L., Otmakhova, N., Carpenter, S., Riahi, S., Asrican, B., and Lisman, J. (2004). Forskolin-induced LTP in the CA1 hippocampal region is NMDA receptor dependent. J Neurophysiol 91, 1955-1962.

Palazzo, A.F., Joseph, H.L., Chen, Y.J., Dujardin, D.L., Alberts, A.S., Pfister, K.K., Vallee, R.B., and Gundersen, G.G. (2001). Cdc42, dynein, and dynactin regulate MTOC reorientation independent of Rho-regulated microtubule stabilization. Curr Biol 11, 1536-1541.

Perlson, E., Jeong, G.B., Ross, J.L., Dixit, R., Wallace, K.E., Kalb, R.G., and Holzbaur, E.L. (2009). A switch in retrograde signaling from survival to stress in rapid-onset neurodegeneration. J Neurosci 29, 9903-9917.

Tagnaouti, N., Loebrich, S., Heisler, F., Pechmann, Y., Fehr, S., De Arcangelis, A., Georges-Labouesse, E., Adams, J.C., and Kneussel, M. (2007). Neuronal expression of muskelin in the rodent central nervous system. BMC Neurosci 8, 28.